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SOME BIOCHEMICAL ASPECTS OF THE IMMUNE MACROPHAGE

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SUMMARY.—Some of the biochemical properties of mouse peritoneal macrophages immune to *Corynebacterium ovis* were characterised. Total cellular protein of immune cells exceeded that of normal phagocytes by 1·85 times. The activities of 7 hydrolytic enzymes, acid phosphatase, β-glucuronidase, Cathepsin D, lysozyme, BPN-ase, MN esterase and aryl sulphatase were measured in lysed cell suspensions and monolayer cultures. Immune macrophages possessed substantially higher levels of these enzymes than did normal cells. No one enzyme was significantly more associated with the development of cellular immunity than another. Resting immune macrophages consumed significantly less oxygen than normal cells required but were twice as active in glycolysis. ATP levels, in agreement, were 5 times higher in normal macrophages whereas ATP-ase activities were equivalent. Normal macrophages were approximately twice as active in protein synthesis measured by the *in vitro* incorporation of ^{14}C L-glycine by monolayer cultures than were immune cells. These results were considered in the light of known morphological differences between the 2 cells noted at the ultrastructural level.

EXPERIMENTAL infection with *Corynebacterium ovis* has been successfully established in the laboratory mouse, in which animal the expression of pathogenesis is very similar to that of the naturally occurring chronic disease of sheep, caseous lymphadenitis. The mouse peritoneal cavity has in particular, provided a useful model for the study of this disease, and for the production of an easily accessible population of macrophages, cells which are known to be involved in the development of resistance to this organism. Macrophages from a previously infected peritoneal cavity possess such immune properties as an enhanced bactericidal capacity and an increased ability to withstand the necrotising action of *C. ovis* (Jolly, 1965*a*, *b*; Hard, 1969*a*). Ultrastructural studies of these immune cells have demonstrated certain morphological differences from the normal, including larger size and increased numbers of lysosomal granules (Hard, 1969*b*).

Most biochemical investigations involving immune macrophages to date, have concerned estimation of their hydrolytic enzymes. Although results generally indicate an increase in lysosomal hydrolases there is disagreement as to whether certain enzymes are more involved in immunity than others. The purpose of the present study was to examine the activity of the enzymes of *C. ovis*-immune cells in order to try to corroborate morphological changes observed at the ultrastructural level, and for comparison with results of earlier work in this field. It

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was further intended to determine if other biochemical differences existed between resting normal and immune peritoneal macrophages.

MATERIALS AND METHODS

Peritoneal cells

Female C57BL and Quackenbush mice (4–6 weeks old) were used. Immunity to virulent *C. ovis* was produced by the i.p. administration of at least 3 injections, one week apart, of the attenuated strain 133A. This clonal substrain was initially recovered from sheep in N.S.W. being attenuated by repeated *in vitro* passage on solid media. The organism retained some degree of pathogenicity in high dosage however. Cultures were initially subbed from lyophilised preparations and grown for 24 hr on tryptose agar slopes. Single cell suspensions were prepared by the method of Jolly (1965*a*) and their approximate concentration estimated by matching with Brown's opacity tubes. The first immunising dose consisted of 10^6 live 133A while subsequent injections contained 10^7 organisms in 0·1 ml. saline.

Peritoneal cells were collected 7 days after the final immunising dose by the method of Whitby and Rowley (1959) in 2·0 ml. of a heparinised nutrient medium. After determining total and differential cell counts the samples were centrifuged at 200 × *g* and the pellets resuspended in triple distilled water and snap-frozen in dry ice for at least 3 days or stored at −20° until required (Cohn and Wiener, 1963). Suspensions from mice with gross peritoneal abscesses, marked splenomegaly or with obvious erythrocyte or polymorphonuclear neutrophil (PMN) contamination were discarded.

Because such peritoneal cell lysates represented a heterogeneous population of cells, reasonably pure samples of macrophages were obtained by culture in Tyrode's nutrient medium on glass cover slips in a 5 per cent CO_2 in air atmosphere at 37°. An equal number of 0·05 ml. drops were pipetted on to acid-washed $\frac{7}{8}$ in. Gold Seal cover slips supported on Pyrex rings. After incubation for 2 hr the cultures were vigorously washed in a jet of saline to remove all unattached cells. The phagocytes were ruptured by pulverising the cover slips in triple distilled water containing 0·1 per cent Triton X 100 with a glass rod. The lysate was snap-frozen for storage at −20°. Sample cultures were stained with May Grunwald Giemsa, in order to determine by light microscopy the approximate number of cells per cover slip and for a differential cell classification.

Biochemical estimations

The chemicals used were always of analar grade.

DNA.—The Bonting and Jones (1957) modification of the indole method (Ceriotti, 1955) was used.

Protein was determined by the bromsulphalein method of Nayyar and Glick (1954) as modified by Bonting and Jones (1957).

Hydrolytic enzymes were assayed by the following methods:

Acid phosphatase.—Sodium β-glycerophosphate substrate was used in the method of Gianetto and de Duve (1955). Inorganic phosphate release was subsequently estimated (Fiske and Subbarow, 1925).

β-glucuronidase.—Talalay, Fishman and Huggins (1945).

Cathepsin D.—Dannenberg and Bennett (1964). Urea denatured haemoglobin was prepared from haemoglobin substrate powder (Rick, 1965).

Lysozyme.—Carson and Dannenberg (1965).

N-benzoyl-DL-phenylalanine-β-naphthol esterase (*BPN-ase*).—By the method of Ravin, Bernstein and Seligman (1954) for chymotrypsin-like enzymes with the modifications recommended by Dannenberg and Bennett (1964).

Esterase hydrolysing β-naphthylacetate (*MN esterase*).—Dannenberg and Bennett (1964).

Aryl sulphatase.—By a method modified from those of Dodgson, Melville, Spencer and Williams (1954) and Roy (1953).

ATP.—This was assayed by the method of Crane and Lippman (1953).

ATP-ase.—For this the incubation mixture consisted of 1·0 ml. of 0·01 M tris HCl buffer, pH 7·5, 0·2 ml. 0·05 M ATP disodium salt and 0·8 ml. cell sample. Incubation at 30° in a water bath was carried out for 30 min. The reaction was stopped by the addition of 2·0 ml. 10 per cent TCA and after brief centrifugation the inorganic phosphate concentration (Fiske and Subbarow, 1925) in the supernatant was estimated.

TABLE I.—*The DNA Content of Normal and Immune Mouse Peritoneal Cells*

Sample number	Normal cells 1	2	3	4	Mean	Immune cells 1	2	3	4	Mean
μg. DNA/10^7 cells	107·66	117·74	74·43	130·00	—	111·00	9·73	57·67	75·88	—
Contaminating PMN or other cells (per cent)	2·0	3·0	13·1	1·0	—	4·4	15·1	25·6	35·7	—
μg. DNA/10^7 cells after correcting for PMN	105·22	120·15	85·62	131·31	110·66. ±9·86	116·11	108·03	77·56	117·94	104·91 ±9·38

Samples 1–3 each represent the combined peritoneal contents of 2 C57BL mice assayed in duplicate; sample 4 is the macrophage monolayer preparation.

TABLE III.—*Lysosomal Hydrolytic Enzyme Content of Peritoneal Cells*

Enzyme	Results Expressed as μg./10μg. of DNA	Total peritoneal cell lysate from Quackenbush mice: Normal (N)	Immune (I)	per cent increase I/N	Total cell lysate from C57BL mice: Normal	Immune	per cent increase I/M	Macrophage monolayer lysate from C57BL mice: Normal	Immune	per cent increase I/N
Acid phosphatase	Phosphate at 2 hr incubation	8·06	15·55	92·9	8·36	14·40	72·3	5·38	16·90	214·1
β-glucuronidase	Phenolphthalein at 2½ hr.	6·69	28·74	329·5	9·15	15·32	67·4	38·0	61·2	61·1
Cathepsin D	I-tyrosine at 2 hr	2·65	5·63	112·5	2·66	3·24	21·8	1·46	2·64	80·8
Lysozyme	Lysozyme at 1 hr	5·29	7·66	44·8	4·85	6·46	33·2	4·54	5·04	122·0
BPN-ase	β-naphthol at 1 hr	1·43	3·56	149·0	2·87	4·69	63·4	0·73	0·82	12·3
MN esterase	β-naphthol at 1 hr	0·93	1·46	46·2	2·16	2·79	29·2	1·18	2·08	80·5
Aryl sulphatase	4-nitro-catechol at 1hr	5·71	13·94	144·1	4·62	7·84	69·7	1·78	4·71	164·6

Respirometry.—Oxygen consumption of live cell suspensions or monolayers in flasks was determined by use of the conventional Warburg apparatus utilising nutrient medium 199 in flasks set up in triplicate.

Glycolysis.—Lactic acid production was determined by a modification (Kessel, Monaco and Marchisio, 1963) of Horn and Bruns' (1956) method which estimates lactate at the critical pH of 10·5 rather than 9·7.

Protein synthesis.—This was assessed by the incorporation of ^{14}C L-glycine by live cells in a method based on that of Sell, Rowe and Gell (1965). Monolayers of macrophages were prepared in acid-washed, crown pyrex flasks. After thorough washing in a jet of normal saline, 2·0 ml. of Eagles' medium plus 20 per cent foetal calf serum containing 0·08 μM ^{14}C L-glycine per ml (specific activity of 0·8μC/μM) were added to each culture. Chloromycetin succinate was included to suppress bacterial contamination. After incubation in a humid 5 per cent CO_2 in air atmosphere at 37° for up to 24 hr. the flasks were washed meticulously with a jet of saline, drained well and 0·8 ml. distilled water and 10·0 ml. scintillator solution (0·4 per cent PPO and 0·01 per cent POPOP in a 2 : 1 mixture of toluene and Triton X 100) added in two stages. Radioactivity was estimated in a Nuclear-Chicago scintillation counter. Background radioactivity for the system was assessed by preparing controls of cells killed by the addition of 0·4 ml. of 40 per cent formalin solution to the monolayers cultures immediately after the labelled amino acid. This resulted in a final concentration of 2 per cent formalin in the flask which caused cell death without significant detachment from the glass surface.

RESULTS

The PMN content of pooled immune mouse suspensions was usually of the order of 8–10 per cent compared with an average of 2 per cent representing eosinophils, PMN and mast cells in normal mouse suspensions. The macrophage content of the immune preparations on the other hand was usually 7–8 per cent higher than that of normal mice, the remainder of the cell population being lymphocyte-like. Differential classification of cover slip monolayers showed normal mouse preparations to contain 97 per cent macrophages and 3 per cent eosinophils, PMN and mast cells collectively while immune cultures consisted of 97 per cent macrophages and 3 per cent PMN.

No significant difference existed between the DNA content of normal or immune macrophages and this parameter was therefore used as a reliable basis for enumerating peritoneal cell concentrations. The results of these estimations on representative samples of pooled cells and macrophage monolayers are shown in Table I.

Results from four duplicate determinations involving pooled groups of several to many mice, indicated that the total protein of immune peritoneal cells exceeded those of the normal mouse by a factor of 1·85 (Table II).

TABLE II.—*Total Protein Content of Normal and Immune Mouse Peritoneal Cells*

Sample	Protein concentration in OD units per 10 μg. DNA at 580 mμ
Normal peritoneal cells	0·205±0·013
Immune peritoneal cells	0·379±0·036

$t_{14} = 2·22; P < 0·05$

In order to compare the various hydrolytic enzyme levels, estimations were carried out in triplicate on a suspension of normal or immune macrophages pooled from 16 mice in each of the two groups. The results have been presented in Table III. In all cases the lysosomal enzyme contents of immune mouse cells were elevated above the values for normal cells. These observations were verified in the pure macrophage monolayer samples. With the exception of BPN-ase and β-glucuronidase, the per cent increases of immune over normal cell enzyme

content of these monolayer cultures were greater than the per cent values for the total peritoneal suspensions. The low values for BPN-ase in lysed monolayer cultures were possibly due to inhibition by the lytic agent Triton X 100 (Table IV).

TABLE IV. *Effect of* 0·1 *per cent Triton X* 100 *on BPN-ase Content of Normal Mouse Peritoneal Cells*

Samples	μg. of β-naphthol release/5×10^6 cells/hr
Triton-free cell-lysate	25·54
Triton-treated cell-lysate	6·03

The percentage increases were higher in the Quackenbush mice than in the C57BL strain for all enzymes tested, although this difference was significant only in the case of BPN-ase and MN esterase. No enzyme was found to be more significantly elevated by antigenic stimulation than any other in these studies.

Estimations of oxygen consumption and lactic acid production in normal and immune macrophages are presented in Fig. 1 and 2. The graphs are constructed

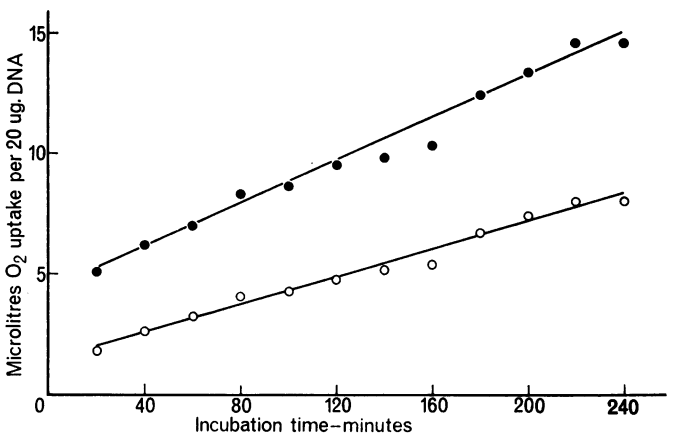

FIG. 1.—Oxygen consumption of monolayers of normal (●———●) and immune (○———○) mouse, peritoneal macrophages. The graph has been compounded from the results of 2 experiments, in each of which triplicate determinations for every point were made.

from the means of at least 2 experiments each performed in triplicate. Oxygen uptake by normal macrophages was 1·55 times that of immune macrophages, while the latter were 2·1 times as active in lactate production as their normal counterparts.

In ATP assay mean values were estimated in 4 samples taken from the pooled peritoneal contents of 12 mice. For ATP-ase estimation duplicate assays were performed on preparation from 4 different mice in each group. The results (Table V) demonstrated that normal macrophages contained 5·2 times the amount of ATP that was present in immune cells. There was no significant difference in ATP-ase content of the 2 cell types.

When the incorporation of ^{14}C L-glycine by normal mouse macrophages in monolayer culture was compared with that of immune cells, it was found that the former possessed 2·14 times the activity of the latter. Considerable variation

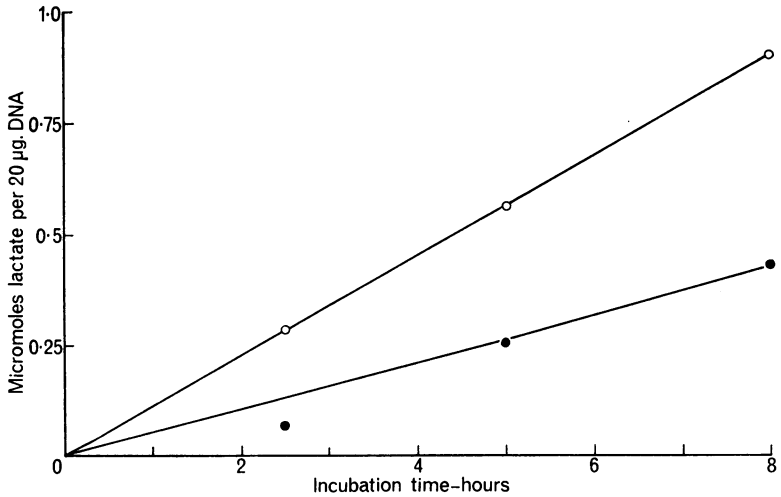

FIG. 2.—Lactic acid production by monolayers of peritoneal macrophages from normal (●———●) and immune (○———○) mice. Each point represents the mean value of 3 determinations.

TABLE V.—*ATP and ATP-ase Content of Normal and Immune Mouse Peritoneal Cells*

Sample	ATP as µM phosphate release	ATP-ase as µM phosphate release
Normal cells	0·244±0·08	5·66±0·06
Immune cells	0·047±0·01	6·14±0·12

in counts was a feature of individual experiments and on one occasion a sample of immune macrophages demonstrated a higher activity than did the normal cells. The mean results from four separate experiments each involving the pooled peritoneal macrophages of 12–16 C57BL mice per group are presented in Table VI. Counts from formalin-killed cells were not significantly higher than

TABLE VI.—*The Incorporation of ^{14}C L-glycine by Cultured Normal and Immune Mouse Macrophages*

Sample	Counts/min./10 µg. DNA
Normal macrophages	4038±900
Immune macrophages	1888±330

$t_{22} = 2{\cdot}32; P < 0{\cdot}05$

those obtained by measuring the radioactivity of the scintillator alone; nor did the uptake by dead cells increase with time. Background values have been subtracted from those presented in Table VI.

DISCUSSION

Light and electron microscopy have previously indicated that macrophages immune to *C. ovis* are larger than normal mouse macrophages. The estimation of total cell protein confirmed this observation.

Similarly the demonstration that immune cells contained significantly higher

quantities of 7 hydrolytic enzymes substantiated the ultra-structural observation of increased numbers and size of lysosomal granules in the former (Hard, 1969*b*). Enzyme estimation of lysed monolayer cultures of peritoneal cells confirmed the results obtained from assay of lysed cell suspensions. The latter represented a heterogeneous population of cells whereas attachment to a glass surface produced a macrophage harvest of high purity. Despite the heterogeneity of cell suspensions the results were expected to be a reliable indication of macrophage enzyme content because the major contaminating cell, the lymphocyte, is known to contain low levels of lysosomal hydrolases in comparison with mononuclear phagocytes.

The fact that all 7 enzymes were elevated in immune macrophages is at variance with some of the previous studies on lysosomal enzyme involvement in cellular immunity. Grogg and Pearse (1952) considered that acid phosphatase was the only enzyme important in the evolution of the resistant mechanism, while Colwell, Hess and Tavaststjerna (1963) working with tuberculosis-resistant rats and guinea-pigs favoured β-glucuronidase. Dannenberg and his co-workers (Dannenberg and Bennett, 1963; Dannenberg and Bennett, 1964; Carson and Dannenberg, 1965; Mizunoe and Dannenberg, 1965); concluded that acid phosphatase, lysozyme and BPN-ase in rabbit macrophages contributed more to cellular immunity of monocytes in tuberculosis than the other hydrolases evaluated. The studies of Cohn and Wiener (1963) involving BCG-induced alveolar macrophages demonstrated increased acid phosphatase, but unaltered Cathepsin D and lysozyme. Heise, Myrvik and Leake (1965) found on the other hand, all three to be elevated in rabbit alveolar macrophages while Saito and Suter (1955) investigating acid phosphatase, β-glucuronidase and Cathepsin D found all three to be implicated in the cellular immunity demonstrated by peritoneal macrophages of BCG-vaccinated mice. When considering these varying results it should be remembered that of the work cited, only the investigations of Saito and Suter involved mouse peritoneal macrophages. Such results do not exclude the possibility of inherent differences between the macrophages of different species, or between peritoneal and alveolar macrophages, as has already been observed by Bennett (1966). De Duve (1963) has postulated that lysosomes may differ widely in their size, structure and enzymic constituents. It has been further suggested that lysosomes are a very heterogeneous group of granules which are selectively disrupted by the appropriate antigen/antibody reaction at the cell surface (Pruzansky and Patterson, 1967). The results of the present study, demonstrating that all of the seven lysosomal hydrolases assayed are potentially capable of playing some part in the immune process by way of increased content in *C. ovis*-resistant peritoneal macrophages, suggests that mouse macrophage lysosomes may be a chemically uniform population of granules. If on the other hand, lysosomes are of varying enzymic composition then the results infer that all granules within the heterogeneous group are unselectively stimulated by exposure to antigen.

Production of lactate was used as the measure of macrophage glycolysis. Cline (1965) has summarised the bulk of evidence which favours the view that the conventional glycolytic pathway is the principle source of leucocyte lactate. The demonstration that immune macrophages rely on glycolysis to a greater extent than normal cells was not unexpected, as it is a feature of the sensitised cells that their culture medium is noticeably more acid after *in vitro* incubation than that supporting cells from the normal peritoneal cavity. A reciprocal dependence on oxygen uptake was observed. Because the glycolytic pathway is consid-

erably less productive of ATP than is aerobic respiration, it was anticipated that the ATP content of immune macrophages might be less than that of normal cells. That the lower ATP levels were not due to an altered activity of ATP-ase was demonstrated by equivalent levels of this enzyme in both normal and immune macrophages. A gradation in respiratory commitments exists in different phagocytic cells. Alveolar macrophages depend for the major part of their energy on oxidative phosphorylation while glycolytic activity is only a minor source. Normal peritoneal macrophages consume considerably less oxygen than alveolar cells but possess a glycolytic capacity exceeding that of PMN. The latter phagocytes consume least oxygen of all (Frei, Borel, Horvath, Cullity and Vannotti, 1961; Pavillard and Rowley, 1962; Dannenberg, Burstone, Walker and Kinsley, 1963; Oren, Farnham, Saito, Milofsky and Karnovsky, 1963; Leake, Gonzalez-Ojeda and Myrvik, 1964; Ouchi, Selvaraj and Sbarra, 1965). Immune macrophages would appear to occupy a position in the scale closer to PMN in oxygen requirements. It has been suggested that the finding of alveolar macrophages in sites of high oxygen tension, and peritoneal macrophages and PMN in sites of low oxygen tension is of some teleological interest (Oren *et al.*, 1963). The fact that both PMN and immune macrophages consume less oxygen than normal peritoneal macrophages may be related to their association with inflammatory exudates, where oxygen tensions are likely to be lower than in normal tissues.

Glycine is the end or by-product of a number of metabolic pathways, and once introduced into the cell, is utilised only for protein synthesis, and to a limited extent in RNA metabolism (Fiegelson and Fiegelson, 1964). Peritoneal macrophages possess a high turnover of protein and RNA but nevertheless have no net requirement for amino acids as the re-utilisation of the breakdown products of RNA and protein degradation is so efficient. Amino acid in the medium is utilised in protein synthesis however (Harris and Watts, 1958; Watts and Harris, 1959). The finding that normal cells are nearly twice as active as immune cells in incorporating radioactive glycine can be interpreted in several ways. The resting immune macrophage being a fully differentiated cell may have a limited requirement for further large scale *de novo* protein synthesis, in contrast to the normal macrophage which is more able to respond to environmental stimuli, because of its potential for differentiaton to the immune state. This does not exclude the fact that immune macrophages under suitable *in vivo* conditions may transform to epithelioid or giant cells (Sutton and Weiss, 1964). Normal macrophages may have been able to incorporate more labelled glycine than immune cells because of a higher pinocytotic activity. Ultrastructural observations suggested that the normal mouse peritoneal macrophage possessed a greater abundance of pinocytotic vesicles than did *C. ovis*-stimulated cells (Hard, 1969*b*). ATP plays a major role in the source of energy for vesicle formation in macrophages (Cohn, 1966), and the lower ATP content demonstrated in immune phagocytes might also infer a lower pinocytotic activity than in normal cells. Pinocytotic vesicle formation is markedly increased when normal peritoneal macrophages are incubated in medium with increasing serum content (Cohn and Benson, 1965*a*, *b*). It is likely that the ability of normal cells to respond in such a way to cultural conditions might exceed that of the immune cell, which is already in an advanced state of maturation.

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